Journal of the Lepidopterists' Society
43(3), 1989, 217–228

GENETIC DIFFERENTIATION AMONG CALIFORNIA POPULATIONS OF THE ANISE SWALLOWTAIL BUTTERFLY, *PAPILIO ZELICAON* LUCAS

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ABSTRACT. The anise swallowtail butterfly, *Papilio zelicaon* Lucas, is widely distributed in California. California *zelicaon* are composed of low- and high-elevation ecotypes defined by host-plant preference and diapause physiology. Electrophoretic-genetic surveys of 14 loci over 10 populations (157 samples total) demonstrate great similarity among these ecotypes, suggesting that their adaptive differences may be defined by a small number of loci rather than broad genomic differentiation.

Additional key words: ecotypes, electrophoresis, Papilionidae.

The anise swallowtail butterfly, *Papilio zelicaon* Lucas, is native to western North America, where it is widely distributed (Tyler 1975). In central California, *zelicaon* is found in a wide variety of habitats from sea level to tree line (Table 1, Fig. 1). Populations at the same latitude exhibit diapause phenologies from univoltine (one generation/yr) to multivoltine (up to four/yr) as a function of habitat elevation, length of growing season, and larval host plant (Sims 1979).

Populations in the Coast Range and the Sierra Nevada above 400 m primarily utilize native Umbelliferae including *Lomatium*, *Angelica*, and *Cymopterus*. These native plants are available to *zelicaon* larvae from spring to midsummer when the onset of hot, dry weather renders the leaves too hard and dry for the larvae to ingest. These populations are univoltine, though in the montane Sierra a second generation occasionally occurs (Sims 1979, Shapiro unpubl.).

Lowland populations (below 400 m) today feed almost exclusively on sweet fennel (*Foeniculum vulgare* Miller, Umbelliferae), which is common throughout coastal and interior lowland California (Munz 1970), and also on orange (*Citrus sinensis* Osbeck, Rutaceae) which has been grown commercially in California since 1841 (Opitz & Platt 1969). Both plants are available to *zelicaon* 8–12 months per year, allowing these populations to breed continuously (Sims 1983). Fennel and orange were introduced to California by Spanish missionaries in the 18th Century (Hutchinson 1969). Both produce natural compounds similar to those in native Umbelliferae (Dethier 1941). Before this *zelicaon* was presumably univoltine, being limited by ephemeral host plants at low elevations and the short growing season in the mountains (Sims 1983). The introduction of these perennial host plants probably

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enabled *zelicaon* to "switch" its ovipositional preference to the introduced plants or to disperse to areas where only the introduced species were available, or both. This, in turn, allowed multivoltinism to evolve in the lowland areas where these plants are abundant (Shapiro & Masuda 1980, Sims 1983).

Papilio zelicaon populations are consistent with the ecotype concept first proposed by Turesson (1922) for hawkweed (*Hieracium*, Asteraceae): plants from different habitats were shown to be phenotypically distinct even when grown under identical conditions, thus demonstrating a genetic basis for the differences.

Using wild *zelicaon* and a laboratory strain selected for nondiapause, Sims (1979) demonstrated that univoltine *zelicaon* populations have significantly higher diapause incidence (photophase required to induce diapause) and intensity (duration of chilling needed to terminate diapause) than multivoltines, and that this phenomenon is genetically based. Sims (1983) showed that incidence and intensity are polygenically inherited, with intensity being affected by maternal phenotype. These characteristics are maintained under varied environmental regimes (Sims 1979, Shapiro unpubl.).

The present study began as an attempt to use electrophoretic analysis to determine whether orange-feeding *zelicaon* in northern California evolved independently of orange-feeding *zelicaon* in southern California, or had been introduced inadvertently from the south. *Papilio zelicaon* was reported as an orange pest as early as 1909 near Visalia, Tulare Co. (Coolidge 1910), and in the 1960's near Chico, Butte Co. (Shapiro unpubl.). This study also investigates the degree to which differentiation of *zelicaon* into low- and high-elevation diapause ecotypes is reflected by electrophoretically detectable genetic variation.

Materials and Methods

Electrophoresis, a commonly used method in biochemical systematics, is based on the movement of charged particles under the influence of an electrical field (Ferguson 1980). Proteins carry a net electrical charge depending on amino acid structures and environmental pH. The rate at which proteins migrate through a support medium is related to their size and shape and is proportional to net charge. Different proteins with different electrophoretic properties migrate at different rates under identical test conditions.

Differential migration of homologous proteins is detectable and of special interest in biochemical systematics. Such differentiation is presumed to reflect differences in nucleic acid sequences that encode proteins. The degree of electrophoretically detected differentiation is thought to reflect the extent of evolutionary divergence between sam-

FIG. 1. Location of populations studied.

pled taxonomic groups, although the structural proteins studied represent only one segment of the overall genome.

Adult *zelicaon* were collected during 1984 and 1985 flight seasons at the sites in Table 1. Captured specimens were frozen live and stored at −70°C to prevent protein denaturation.

In preparation for analysis, thoraces were excised and homogenized in 600 μl of glass-distilled water with a Teflon-coated tissue grinder. Homogenate was absorbed onto 2 × 9 mm wicks of #3 Whatman paper and applied to the gels. Horizontal slab gels were made with Sigma starch and were prepared and run for 5 h using methods described in Ayala et al. (1972, 1974a).

After running, gels were cut into four 2-mm-thick slices so that each

TABLE 1. California field site characteristics (Shapiro unpubl.).

Population	Location	Elevation (m)	No. generations/ year	Larval host plant	Habitat
Gazelle	Siskiyou Co.	838	1–2	*Angelica arguta, Conium*	Great Basin, grassland, farmland, sloughs
Washington	Nevada Co.	1220	1	*Lomatium*	Sierran W slope, serpentine
Hemet	Riverside Co.	487	>1	*Citrus*	S. California desert, orchards
Orland	Butte Co.	67	>1	*Citrus*	Central Valley orchards
Butts Canyon	Napa Co.	457	1	*Lomatium*	North Coast Range, serpentine
Suisun	Solano Co.	9	>1	*Foeniculum vulgare*, rarely *Cicuta*	Central Valley levees, disturbed areas, near freshwater and tidal marshes
Blue Ridge, +Gates Canyon	Solano Co.	60–762	>1	*Foeniculum vulgare*	Vaca Hills, canyon, riparian
Rancho Cordova	Sacramento Co.	9	>1	*F. vulgare*, rarely *Conium*	Central Valley, riparian forests and gravel beds
Castle Peak	Nevada Co.	2743	1	*Cymopterus*, Umbelliferae spp.	Sierran, alpine
Auburn	Placer Co.	366	1–>1	*Foeniculum vulgare*, Umbelliferae spp. in canyons	Sierran W slope; univoltines: canyon, multivoltines: vacant lots in town

TABLE 2. Enzymes assayed.

Enzyme	Abbreviation	Buffer*
Phospho-glucose isomerase	PGI	REG
Aldolase	ALDO	REG
α-Glycerophosphate dehydrogenase	αGPD	REG
Glutamate-oxaloacetate transaminase	GOT-1	REG
Hexokinase	HK-1	REG
Phospho-gluco mutase	PGM	REG
Fumarase	FUM-2	REG
Mannose phosphate isomerase	MPI	REG
Malic enzyme	ME-1	JRP
Glucose-3-phosphate dehydrogenase	G3PD	DH
Glucose-6-phosphate dehydrogenase	G6PD	DH
Hydroxybutyrate dehydrogenase	HBDH	DH
Esterase	EST-1	DH
	EST-2	DH

* REG: Gel buffer—9 mM Tris, 3 mM citric acid, pH 7.0. Electrode buffer—135 mM Tris, 45 mM citric acid. JRP: Gel buffer—76 mM Tris, 5 mM citric acid, pH 8.65. Electrode buffer—300 mM boric acid, 60 mM NaOH. DH: Gel and electrode buffer—8.7 mM Tris, 8.7 boric acid, 1 mM EDTA, 1 mM β-NAD^+, pH 9.0.

sample was tested for four enzymes. Table 2 lists the enzymes assayed. Specific staining systems and gel fixation techniques are described in Ayala et al. (1972, 1974a).

Fixed gels were scored after each run using a light box. Loci were characterized and interpreted as for Pieridae, for which the genetic basis of the electrophoretic banding patterns has been demonstrated in an extensive breeding program (Geiger 1981, Burns & Johnson 1971). Electromorphs were recorded as distance (mm) migrated from the origin.

Electromorph frequencies (considered as allelic frequencies) were used to calculate I, a statistic of genetic identity between taxa (Nei 1972), for all pairwise comparisons of populations. I-values were analyzed using the UPGMA method of cluster analysis (Ferguson 1980).

G-tests (Sokal & Rohlf 1981) were performed on genotype frequencies in the populations represented by large samples ($\geq$14 individuals) to determine whether observed frequencies for each population were consistent with Hardy-Weinberg equilibrium, and whether all populations can be considered to represent a single panmictic population.

RESULTS

Table 3 shows the electromorph frequencies for each population. Of the 14 loci assayed, three are polymorphic: PGI, PGM and MPI.

Results of *G*-tests are displayed in Table 4. Most loci exhibit Hardy-Weinberg equilibrium. However, weighted-average results show that among the populations examined, *zelicaon* does not exhibit Hardy-Weinberg equilibrium and cannot be considered a single, panmictic population. Genotype frequencies are shown in Table 7.

TABLE 3. Electromorph frequencies in 10 *Papilio zelicaon* populations in California.

		Population (n)										
Locus	Allele	Gazelle (8)	Washington (6)	Hemet (19)	Orland (19)	Butts Canyon (14)	Suisun (25)	Blue Ridge (7)	Rancho Cordova (16)	Castle Peak (35)	Auburn (8)	Overall (157)
PGI	13	0	0	0	0.05	0.20	0	0	0.111	0.128	0	0.14
	7	1.0	1.0	1.0	0.95	0.80	1.0	1.0	0.89	0.87	1.0	0.86
ALDO	10	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
αGPD	23	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
GOT-1	27	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
HK-1	40	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
PGM	32	0.50	0	0.26	0.10	0.18	0.23	0.25	0.29	0.23	0.15	0.22
	26	0.50	0.20	0.21	0.47	0.18	0.46	0.25	0.35	0.28	0.23	0.33
	23	0	0.40	0.32	0.40	0.45	0.18	0.33	0.23	0.35	0.46	0.31
	20	0	0.40	0.21	0.03	0.18	0.14	0.17	0.03	0.13	0.15	0.13
FUM-2	12	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
MPI	44	0	0	0	0.06	0	0.09	0	0	0.02	0	0.03
	40	0	0	0	0.06	0	0.09	0	0	0.08	0	0.04
	37	0	0	0.30	0.20	0.43	0.18	0.40	0.09	0.30	0	0.22
	33	0.33	0.50	0.57	0.60	0.50	0.64	0.60	0.73	0.52	0.40	0.56
	28	0.67	0.50	0.13	0.06	0.07	0	0	0.18	0.80	0.60	0.15
ME-1	20	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
G3PD	7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
G6PD	7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
HBDH	6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
EST-1	22	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
EST-2	14	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

TABLE 4. *G*-test of genotype frequencies in populations ≥14.

Locus (df)	G-statistics for populations						
	Hemet	Orland	Butts Canyon	Suisun	Rancho Cordova	Castle Peak	Average
PGI (1)	0	4.194*	6.605*	0	1.010	2.966	12.776*
PGM (6)	24.349*	20.526*	4.502	13.382*	21.838*	6.846	48.90*
MPI (10)	3.224	3.918	1.582	1.976	10.606	10.334	30.926*

* $P \leq 0.05$. $G = 2\Sigma\ \text{OBS} \ln\frac{\text{OBS}}{\text{EXP}}$; $df = \frac{1}{2}(n^2 - n)$ (Ferguson 1980).

Table 5 shows the I-values calculated from electromorph frequencies for each pairwise comparison of populations. The average I-value is 0.980 ± 0.139, indicating a very high level of genetic similarity.

To highlight differentiation without altering phenetic clustering of populations, I-values were recalculated using only data for the three polymorphic loci (Table 6). By excluding the background of monomorphic loci, a more useful graphic analysis can be generated. The UPGMA dendrogram derived from these data is shown in Fig. 2.

DISCUSSION

The high identity values (Table 5) are consistent with similar studies on other insects. Ayala et al. (1974b) reported $\bar{I}$ of 0.970 ± 0.006 for *Drosophila willistoni* populations sampled throughout Central and South America, substantiating previous conclusions about their relatedness based on reproductive relations. Brussard et al. (1985) surveyed genetic identity findings comparing 14 insect taxa (including 3 Lepidoptera), and reported $\bar{I}$ of 0.97 for local populations. It is likely that these values are conservative. Mutants of crucial glycolytic or catabolic enzymes are likely to be eliminated by selection (Bell 1976; Turner 1974; Zera et al. 1985).

Reproductive relations in *zelicaon* are not clear. Breeding trials among

TABLE 5. Genetic identity I-value matrix using 14 loci ($\bar{I}$ = 0.980 ± 0.139).

	Washington	Hemet	Orland	Butts Canyon	Suisun	Blue Ridge	Rancho Cordova	Castle Peak	Auburn
Gazelle	0.97	0.96	0.96	0.95	0.96	0.96	0.98	0.97	0.98
Washington	—	0.98	0.97	0.98	0.97	0.98	0.98	0.98	0.99
Hemet		—	0.99	0.99	0.99	0.99	0.99	0.98	0.97
Orland			—	0.99	0.99	0.99	0.99	0.98	0.97
Butts Canyon				—	0.99	0.99	0.99	0.99	0.97
Suisun					—	0.98	0.99	0.98	0.96
Blue Ridge						—	0.99	0.99	0.97
Rancho Cordova							—	0.99	0.98
Castle Peak								—	0.98
Auburn									—

TABLE 6. I-value matrix using only the three polymorphic ($\bar{I}$ = 0.867 ± 0.100) loci.

	Washington	Hemet	Orland	Butts Canyon	Suisun	Blue Ridge	Rancho Cordova	Castle Peak	Auburn
Gazelle	.765	.713	.707	.656	.723	.676	.807	.730	.853
Washington	—	.855	.830	.842	.795	.812	.849	.850	.950
Hemet		—	.953	.962	.960	.982	.953	.985	.784
Orland			—	.930	.974	.953	.957	.973	.783
Butts Canyon				—	.900	.984	.901	.979	.808
Suisun					—	.952	.966	.964	.719
Blue Ridge						—	.942	.990	.754
Rancho Cordova							—	.757	.822
Castle Peak								—	.808
Auburn									—

multivoltines from northern and southern California and the Central Valley show these populations to be intercompatible (Shapiro unpubl.). Alternatively, Sims (1983) suggests that univoltines and multivoltines are not fully intercompatible because of male-biased hybrid broods. However, control (within-population) data are not available in adequate numbers to validate this conclusion.

While I-values suggest that all *zelicaon* populations are conspecific, the weighted-average *G*-tests show that *zelicaon* is, of course, not panmictic over its entire range. Figure 2 suggests that populations can be clustered on the basis of geographic proximity.

Gazelle (Shasta Valley) is most genetically dissimilar, and is probably more geographically isolated as well. Washington and Auburn are Sierran west slope univoltines. Orland, Suisun and Rancho Cordova are Central Valley multivoltines. Castle Peak, Butts Canyon, Blue Ridge, and Hemet represent univoltine and multivoltine populations in a mixture of very diverse ecological contexts.

The germinal issue is to what degree populations are reproductively isolated by host-plant selection and physical distance. Certainly, the breeding trials and high I-values suggest that all *zelicaon* populations are potentially intercompatible. However, voltinism may be a genetically heritable trait that divides *zelicaon* into low- and high-elevation ecotypes (Clarke & Sheppard 1970).

Our data support Sims's (1983) contention that *zelicaon* diapause physiology and host-plant selection are highly plastic. The clustering of the orange-feeding Orland population with other Central Valley populations rather than with Hemet implies that the northern and southern orange-feeders evolved separately. While fennel is abundant in lowland areas, and is heavily used (Shapiro 1974a, 1974b), the use of orange may allow *zelicaon* to increase its range despite the inferiority of orange as a host plant (Masuda 1981).

TABLE 7. Observed genotype frequencies for the three polymorphic loci in populations where n ≥ 14.

Genotype	(Electromorph-electromorph)	Populations					
		Hemet	Orland	Butts Canyon	Suisun	Rancho Cordova	Castle Peak
PGI	13-13	0	0	0.14	0	0	0.13
	13-7	0	0.05	0.07	0	0.13	0.11
	7-7	1.00	0.95	0.80	1.00	0.89	0.86
PGM	32-32	0.21	0.11	0	0.08	0	0.06
	32-26	0	0	0	0.08	0.31	0.11
	32-23	0.05	0.05	0.17	0.08	0.19	0.11
	32-20	0	0	0.17	0.08	0	0.09
	26-26	0.11	0.21	0	0.21	0.06	0.09
	26-23	0.26	0.53	0.33	0.25	0.19	0.23
	26-20	0	0	0	0.42	0.13	0.09
	23-23	0.21	0.05	0.17	0.42	0	0.14
	23-20	0.05	0	0.17	0	0.13	0.03
	20-20	0.11	0.05	0	0.13	0	0.06
MPI	44-44	0	0	0	0	0	0
	44-40	0	0	0	0	0	0
	44-37	0	0	0	0	0	0
	44-33	0	0.10	0	0.14	0	0.03
	44-28	0	0	0	0	0	0
	40-40	0	0	0	0	0	0
	40-37	0	0	0	0	0	0
	40-33	0	0.10	0	0.14	0	0.10
	40-28	0	0	0	0	0	0
	37-37	0.11	0.10	0.13	0	0	0.13
	37-33	0.32	0.20	0.50	0.29	0.10	0.29
	37-28	0.05	0	0	0	0	0.03
	33-33	0.42	0.40	0.25	0.43	0.70	0.32
	33-28	0.05	0.10	0.13	0	0	0.67
	28-28	0.05	0	0	0	0.20	0.03

Butts Canyon (North Coast Range serpentine) and Castle Peak (Sierran volcanic alpine) probably represent relict *zelicaon* populating rocky, unforested environments with endemic host plants. Other Lepidoptera are known to be similarly disjointly distributed between the Coast Range serpentines and the alpine Sierra; *Papilio indra*, *Pieris sisymbrii*, and *Euchloe hyantis* all occur obligately in these areas with few or no intervening populations (Shapiro unpubl.). Clustering of Blue Ridge (east of the Vaca Hills, the easternmost part of the Inner North Coast Ranges in Yolo and Solano cos.) with these postulated relict populations rather than with other multivoltines in the Central Valley is especially interesting. *Papilio zelicaon* was not seen in the Vaca Hills during summer in field studies initiated by Shapiro (unpubl.) in 1972. Males were seen on the ridge-tops, but only in spring coinciding with such behavior on Coast Range serpentines to the north. At this time, the site had one patch of 10 fennel plants. In 1975, females were observed

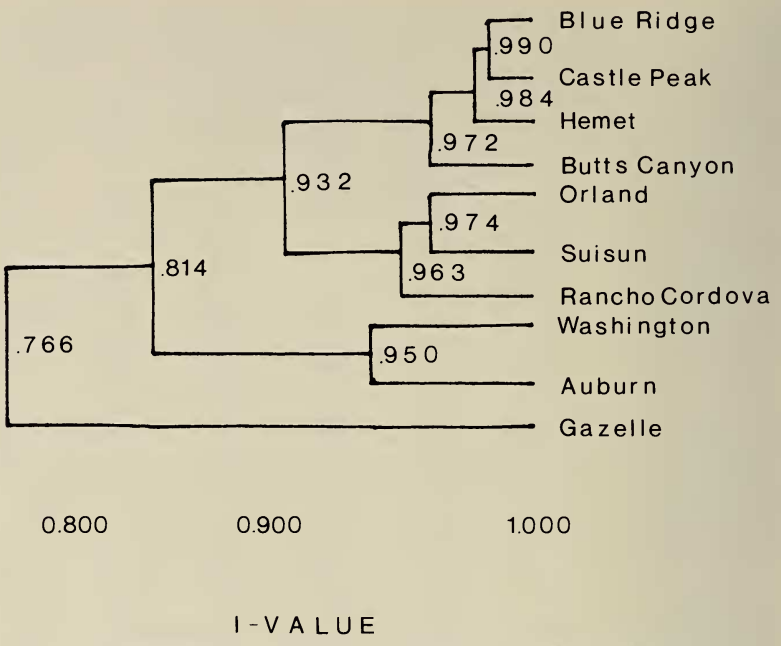

FIG. 2. Phenogram of *P. zelicaon* populations (UPGMA; Ferguson 1980).

ovipositing on fennel. By 1978, fennel was spreading rapidly in disturbed areas and *zelicaon* showed evidence of four generations in one year. Presently, there are over 500 fennel plants along three miles of road in this area, and it continues to spread. It has been presumed that the multivoltine Vaca Hills *zelicaon* are upslope colonists from multivoltine Central Valley populations. Our study suggests, rather, that they are at least partially downslope colonists from univoltine ridge-top (Coast Range) populations. If this is the case, they have very rapidly evolved multivoltinism, apparently as an adaptation to the spread of fennel. This supports the plasticity of host plant- and diapause-"switching" proposed by Sims (1983) to explain the evolution of multivoltinism. Certainly, *zelicaon* is physically capable of having colonized these canyons from the Coast Range. Shields (1967) demonstrated that *zelicaon* is a hilltopping species; adult males and receptive females congregate on summits to mate, thereby promoting gene flow among neighboring populations. Shields determined that adults are capable of traveling several km per day.

Studies by Ehrlich and Raven (1969) and Endler (1973) suggest that populations undergoing sufficiently strong divergent selection will differentiate despite the counter-effects of continuous gene flow. This has been observed in wild Lepidoptera with populations showing differ-

entiation in metrical traits as a result of differential selection, despite close proximity and gene flow (Creed et al. 1959, Clarke & Sheppard 1962). If gene flow along the Coast Range ridge-tops has been continuous, the Vaca Hills population has not only become multivoltine within three years time, but has done so with constant influx of univoltines from the Coast Range. Multivoltinism may be evolving through hybridization, or through selection. Multivoltinism shortens generation time and should, all other factors being equal, be selectively advantageous.

Wright (1943a, 1943b) theorized that a continuously distributed species exposed to different conditions of selection would differentiate if subdivided into partially isolated "islands" separated through inbreeding or limited dispersal ability. *Papilio zelicaon* is certainly distributed throughout habitats with different selection conditions and appears to be sufficiently vagile to be essentially continuous in distribution throughout major portions of its range. More finely focussed studies of nonglycolytic enzymes and mark-release-recapture studies on movement would help to determine the size and location of hilltopping regions and the appropriateness of Wright's "island" models to *zelicaon*.

Papilio zelicaon is composed of low- and high-elevation ecotypes defined by host-plant preferences and diapause physiology. These traits may be determined by a relatively small number of loci that are under strong selection pressure and whose distribution is not reflected by electrophoretically accessible glycolytic enzyme loci, which show great genetic similarity among populations.

ACKNOWLEDGMENTS

We thank F. J. Ayala for use of laboratory facilities, Hansjürg Geiger and P. Ward for helping us interpret electrophoretic data, and John Emmel for the Hemet specimens; also Douglas Engfer for developing software to compute I-values. This study forms part of California Agricultural Experiment Station Project CA-D*-AZO-3593, "Host Switching by the Anise Swallowtail," A. M. Shapiro, Principal Investigator.

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Received for publication 5 July 1988; accepted 26 May 1989.